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(54) Title: ANTIBODY

(57) Abstract: The present invention relates to a functionally active binding fragment of and an antibody that binds at least two strains of Venezuelan equine encephalitis virus, pharmaceutical compositions comprising fragments or antibodies and methods of their use.

Antibody

The *Alphavirus* Venezuelan equine encephalitis virus (VEEV) is a single stranded, positive-sense RNA virus maintained in nature in a cycle between small rodents and mosquitoes. Six serogroups (I-VI) are currently recognised within the VEEV complex. Spread of epizootic strains of the virus (IA/B and IC) to equines leads to a high viraemia followed by lethal encephalitis and lateral spread to humans. In the human host, VEEV can produce a febrile illness followed in a small proportion of cases by severe encephalitis. Equine epizootics may lead to widespread outbreaks of human encephalitis involving thousands of cases and hundreds of deaths. Viruses in other serogroups do not appear to be equine-virulent and persist in a stable enzootic cycle. Natural transmission of enzootic viruses to humans is rare but may be associated with severe disease.

Epizootic VEEV can be controlled by the immunisation of equines with the attenuated vaccine strain TC-83. Although TC-83 is solidly protective in equines and has a good safety record, in humans it fails to produce protective immunity in up to 40% of recipients and is reactogenic in around 20% of recipients. There have also been reports that the vaccine is potentially diabetogenic and teratogenic. Consequently, TC-83 is no longer available for human use. Both epizootic and enzootic strains of VEEV are infectious for humans by the airborne route and have been responsible for a number of laboratory infections.

In the absence of a suitable vaccine, antiviral therapies which are effective in prophylaxis and treatment of VEEV infection are required. There is evidence to suggest that protection against VEEV requires high antibody levels and, in the case of airborne infection, the presence of antibody on the mucosal surface of the respiratory tract (Phillpotts et al., 2002). Previous studies have shown that monoclonal antibodies can protect against VEEV and are effective against disease even when administered 24h after exposure (Hunt et al., 2006; Phillpotts et al., 2002; Phillpotts, 2006). Monoclonal antibodies, however, tend to have narrow specificities which limit their use as antiviral therapies. A new broadly reactive antibody which

would have the potential to protect against exposure to a range of VEEV strains, is required.

The present disclosure provides a functionally active binding fragment of an antibody that binds at least two strains of Venezuelan equine encephalitis virus.

The antibodies and fragments of the disclosure are thought to be suitable for treating infection with several strains of VEEV and/or preventing infection with the same, or at least ameliorating the severity of the infection.

Brief Description of the Figures

Figure 1 Shows reactivity of phagemid clones to a wide range of VEEV strains

Figure 2 Shows an annotated amino acid sequence of a single chain variable region labelled CUF37-2a

Figure 3 Shows reactivity of CUF37-2a to multiple VEEV strains

Figure 4 Shows CUF37-2a reacts with the VEEV E2 glycoprotein

Figure 5 Shows data that CUF37-2a protects against VEEV disease when administered 24h prior to challenge

Figure 6 Shows data indicating enhanced levels of protection against VEEV by prior treatment with increasing amounts of CUF37-2a

Antibodies comprise two heavy chains, each comprising a constant region and a variable region. These heavy chains are linked by disulfide bridges at the so-called hinge region. In addition each heavy chain is linked to a light chain (also comprising a constant region and variable region) through further disulfide bridges in an arrangement that is often referred to as forming an overall "Y" shape. Each variable region has three complementary determining regions (CDRs). Together the variable region of a light chain and heavy chain define the binding specificity of the antibody for the target.

A variable region from a heavy chain or a light chain can be considered as a basic functional binding unit of antibody and is sometimes referred to as a domain antibody. Alternatively a variable region from a heavy chain and light can be

associated together, for example by covalent bonds to provide what is referred to as a single chain variable fragment (scFv), and comprises three CDRs from the heavy and three CDRs from the light chain (nominally referred to as H1, H2, H3 for the heavy chain and L1, L2 and L3 for the light chain), in the same way as a complete antibody. The single chain variable fragment may have similar binding characteristics to the corresponding full antibody but often the kinetics *in vivo* of the single chain variable fragment are not the same as the full antibody, and the fragment may be metabolised faster than the corresponding full antibody. Nevertheless, this phenomenon can be compensated for by, for example PEGylating the fragment (see also Knauf et al J. Biol Chem 263: 15064-15070 (1988)). US patent application publication No: 2003/0021790 describes conjungates of antibodies and one or more non-proteinaceous polymers, with an improved half life.

The association of one complete heavy chain and one complete light chain is known as a "single chain". Single chains, that is to say a heavy chain and light chain associated together can be employed as can full antibodies or other permutations/variations on any of the above.

In one embodiment the functionally active binding fragment comprises a variable region, for example from a heavy chain or a light chain optionally associated with a portion of a constant region.

In one embodiment the functionally active binding fragment comprises a single chain variable fragment that is a variable region from a heavy chain and a variable region from a light chain, which may, for example be linked covalently.

In one embodiment the functionally active fragment comprises a variable region from a heavy chain, a variable region from a light chain and constant region from a heavy and/or light chain from above the hinge region (referred to herein as a fab fragment).

In one embodiment the functionally active binding fragment comprises a single chain.

In one embodiment the invention provides an antibody comprising:
a variable region from a heavy or light chain,
a single chain variable fragment,
a fab fragment and/or
a single chain as defined herein.

Antibody as used herein refers to a complete antibody or a conjungate comprising a complete antibody.

An antibody fragment as used herein refers to an entity which is less than a full antibody, for example a variable region from a heavy and/or light chain, a single chain variable region, a Fab fragment, a variable region and a portion of a constant region, a heavy chain, a light chain, a single chain or the like and including conjugates of each of the same.

Functionally binding fragment as used herein refers to a fragment that recognises/binds the same entities or substantially the same entities as the corresponding full antibody, although not necessarily with the same affinity or avidity, but none the less can be used to perform a corresponding function to that of the full antibody.

Any of the embodiments defined herein may comprise a CDR, nominally referred to herein as H1, for example with the sequence **DYYMN** (or this sequence wherein one amino acid has been replaced).

H1 may be located at amino acid residue about 184 to about 188, for example in a sequence such as shown in Figure 2 or an equivalent functional position in a longer or shorter sequence.

Any of the embodiments defined herein may comprise a CDR, nominally referred to herein as H2, for example with the sequence

WIGWIDPENGDTEYAPKFQG (or this sequence wherein one amino acid has been replaced).

H2 may be located at amino acid residue about 200 to about 219, for example in a sequence such as shown in Figure 2 or an equivalent functional position in a longer or shorter sequence.

Any of the embodiments defined herein may comprise a CDR, nominally referred to herein as H3 for example with the sequence **EVGRGTSAY** (or this sequence wherein one amino acid has been replaced).

H3 may be located at amino acid residue about 252 to about 260, for example in a sequence such as shown in Figure 2 or an equivalent functional position in a longer or shorter sequence.

Any of the embodiments defined herein may comprise a CDR, nominally referred to herein as L1, for example with the sequence **KASQDIKSYLS** (or this sequence wherein one amino acid has been replaced).

L1 may be located at amino acid residue about 49 to about 59, for example in a sequence such as shown in Figure 2 or an equivalent functional position in a longer or shorter amino acid sequence.

Any of the embodiments defined herein may comprise a CDR, nominally referred to herein as L2, for example with the sequence **YATTLAD** (or this sequence wherein one amino acid has been replaced).

L2 may be located at amino acid residue about 75 to about 81, for example in a sequence such as shown in Figure 2 or an equivalent functional position in a longer or shorter amino acid sequence.

Any of the embodiments defined herein may comprise a CDR, nominally referred to herein as L3, for example with the sequence **LQHYESPYT** (or this sequence wherein one amino acid has been replaced).

L3 may be located at amino acid residue about 114 to about 122.

The disclosure also extends to embodiments comprising the following combination of CDRs:
H1 and L1, H1 and L2, H1 and L3, H1 and H2, H1 and H3, H2 and L1, H2 and L2, H2 and L3,H3 and L1, H3 and L2, H3 and L3, L1 and L2, L1 and L3, H1 and H2 and L1, H1 and H2 and L2, H1 and H2 and L3, H1 and H2 and H3,H1 and H3 and L1, H1 and H3 and L2, H1 and H3 and L3, H2 and H3 and L1, H2 and H3 and L2, H2 and H3 and L3, H1 and H2 and H3, L1 and L2 and H1, L1 and L2 and H2, L1 and L2 and H3, L1 and L2 and L3, H1 and H2 and H3 and L1, H1 and H2 and H3 and L2, H1 and H2 and H3 and L3, L1 and L2 and L3 and H1, L1 and L2 and L3 and H2, L1 and L2 and L3 and H3, H1 and H2 and H3 and L1 and L2, H1 and H2 and H3 and L1 and L3, H1 and H2 and H3 and L2 and L3,L1 and L2 and L3 and H1 and H2,L1 and L2 and L3 and H1 and H3,L1 and L2 and L3 and H2 and H3, or H1 and H2 and H3 and L1 and L2 and L3, as defined herein.

In one embodiment the invention provides a single chain variable fragment as shown in Figure 2.

In one embodiment the invention provides a fragment such as single chain variable fragment with the corresponding function to the fragment shown in Figure 2 or an antibody with a corresponding function. In one embodiment, where the fragment of the invention forms part of an antibody of the invention, the antibody of the invention is not TC-83. The fragment of the invention may be heterologous to the antibody of the invention.

In one embodiment the antibody or fragment according to the disclosure is linked to a label, which is detectable directly or indirectly, for example a radioisotope,

enzyme, fluorophore or a luminescent substance by means such as chemical cross-linking or genetic manipulation, as appropriate.

In one embodiment the invention provides a fragment such as single chain variable fragment with the corresponding function to the fragment shown in Figure 2 or an antibody with a corresponding function which is linked to a biological reporter system such as an enzyme by means such as chemical cross-linking or genetic manipulation. Alternative labels include a radioisotope, fluorophore or a luminescent substance.

In one embodiment the antibody or fragment thereof reacts with cells expressing the E1 and/or E2 protein that occur on the virus surface as a heterodimer, such as the E2 protein.

The disclosure also extends to sequences with 80% or more, such as 85%, 95%, 96%, 97%, 98% or 99% homology to a sequence herein, for example when the comparison is performed against the full sequence disclosed.

Two sequences are said to be homologous if one sequence has a high enough degree of identity and/or similarity when aligned to the other sequence, that is, they share 80% or more such as 85%, 95%, 96%, 97%, 98% or 99% identity and/or similarity. "Identity" indicates that at any particular position in the aligned sequences, the amino acid residue is identical between the sequences. "Similarity" indicates that at any particular position in the aligned sequences, the amino acid residue is of a similar type between the sequences. For example, amino acid residues can be grouped by their side chains. Glycine, alanine, valine, leucine and isoleucine all have aliphatic side-chains and amino acids in this group may be regarded as similar. Proline, although a cyclic amino acid, shares many properties with the aliphatic amino acids and may also be regarded as being grouped with the other aliphatic amino acids. Another group is the hydroxyl or sulphur containing side chain amino acids. These are serine, cysteine, threonine and methionine. Phenylalanine, tyrosine and tryptophan are grouped together as the aromatic amino acids. Histidine, lysine and arginine are the basic amino acids. Aspartic acid and

glutamic acid are the acidic amino acids and asparagine and glutamine are their respective amides. Also included in these groups are modified amino acids (i.e. non-naturally occurring amino acids) that have side-chains that share similar properties with the naturally occurring amino acids. Members of a particular group can be regarded as being "similar". Swapping one amino acid from a group with another amino acid from the same group is often termed a conservative substitution.

Degrees of identity and similarity can be readily calculated using known computer programs (see Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing. Informatics and Genome Projects, Smith, D. W.,ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part1, Griffin, A. M. , and Griffin, H. G. , eds. , Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991). For example, simple sequence comparisons can be done on web-sites such as the NCBI website: http://blast.ncbi.nlm.nih.gov.uk (version 2.2.18). As used herein, percentages identity or similarities between sequences are measured according to the default BLAST parameters, version 2.2.18. A typical list of default scoring parameters would be blosum 62 matrix; 1,-2 match/mismatch scores; existence: 11 extension: 1 gap costs; conditional compositional score matrix adjustment and species-specific repeats for human.

In one embodiment a fragment is employed and the half-life of the entity employed is increased by forming a conjungate with a non-proteinaceous polymer, for example forming a conjungate with PEG or cholesterol.

The entity employed in the disclosure is not is recombinant, that is to say is synthesised by recombinant techniques or cells prepared by recombinant techniques. Thus whilst the initial antibodies or fragments may have been generated *in vivo* once an antibody entity has been identified as suitable for use according to the disclosure it will generally be prepared by techniques commonly employed for manufacturing/producing antibodies *ex-vivo*, for example in a mammalian cell line.

In one embodiment the antibody or antibody fragment employed is chimeric, that is it comprises sequences from at least two different species, for example human and murine, rodent, porcine, camel, equine, canine, rabbit, sheep or similar. In one embodiment the constant region in the entity may be derived from a human constant region but the CDR region may be derived from a second species such as mouse. Nevertheless individual residues in the CDRs may be changed to render them more acceptable for administration to humans (humanised).

There are different types of antibodies, which may be employed in the disclosure such as IgM, IgG, IgA and IgD and sub-types thereof, namely G1, G2, G3, G4, A1 and A2.

In one embodiment the antibody or fragment thereof is IgG, for example IgG2, such as IgG2a.

In one embodiment of the disclosure a heavy chain is a mu, gamma, delta or epsilon isotope.

In one embodiment of the disclosure a light chain is a kappa or lambda isotope.

The disclosure provides antibodies or fragments thereof that are at least bispecific, that is to say that they recognise at least two strains of the VEEV, such as three, four or five strains of VEEV, in particular all known strains of VEEV capable of causing an epidemic in animals (eg IA/B and IC), especially viruses from subtypes IA/B, IC, ID, IE, IF, II, IIIA, IV, V and VI or all known strains of VEEV.

In one embodiment the antibody or fragment thereof according to the disclosure is suitable for the treatment or prophylaxis of a mammal, such as a human and/or equine. As used herein, "a patient" refers to a mammal.

Processes for the generating and selection of antibodies are well known, for example antigen, in this case virus, or mixtures thereof are injected into a host.

Hybridomas can be generated or alternatively B cells with the relevant sequences presented on their surface can be isolated, screened and selected. Phage display is another method often employed in screening antibodies, where the relevant sequence is presented on the surface of certain cells. Generally once a suitable antibody is selected from initial screening it is usually sequenced and prepared in a purified form for more vigorous testing, optimization and/or humanisation, as appropriate.

Mammalian cells are often the most suitable for the preparation of antibodies and fragments thereof because they produce antibodies or fragments thereof with the suitable folding, which of course can be important to *in vivo* activity.

The disclosure extends to methods of preparing, screening, identifying and/or purifying an antibody as defined herein.

Whilst not wishing to be bound by theory it is thought that the antibody of the disclosure or the fragment thereof is effective by a passive mechanism ie it binds the circulating virus, thereby targeting the virus for destruction. This may also give the body the opportunity to generate antibodies *de novo* as a result of being exposed to the virus.

The antibodies of the disclosure or fragment thereof may be administered before exposure to the virus, for example 24 hours, one week, or month before exposure to the virus (thereby allowing the antibody or fragment thereof to get into the circulation of the individual) or may be administered after exposure to the virus such as within 4, 8, 12, 24 or 36 hours or one week of exposure. Alternatively the administration may be before exposure to the virus and also after exposure to the same.

The pathogenesis of VEEV disease in mice and humans is believed to be similar and in mice the virus usually enters the central nervous system two or three days after peripheral inoculation (Bennett et al., 2001). After airborne infection there is the additional possibility that virus may multiply in the olfactory neuroepithelium

and thereby gain direct access to the olfactory nerve and brain. Thus, there is limited time available for antivirals to be administered after exposure to VEEV if they are to be used as therapeutics rather than as prophylactics. When other monoclonal antibodies were used as post-exposure antiviral therapies for VEEV, they were only effective when administered 24h after infection (Hunt et al., 2006; Phillpotts et al., 2002) and not at 48h (Hunt et al., 2006) or 72h (Phillpotts et al., 2002). Antivirals therefore may need to be administered quickly enough after infection to prevent VEEV from accessing the brain or, alternatively, antivirals that are able to cross the blood-brain barrier are required to block viral replication in the brain.

In one embodiment multiple administrations of the antibody or fragment thereof are employed, for example with 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 day, 3, 4, 5, 6 or 7 weeks between administrations.

In one embodiment the antibody is administered as a parenteral formulation, for example by infusion or vaccination (such as intradermal, subcutaneous, intramuscular, intravenous). Other formulations such as intranasal formulations are also envisaged.

Thus the disclosure provides a pharmaceutical formulation comprising an antibody according the disclosure or a fragment thereof and an excipient.

The formulation may, for example may be lyophilised for reconstitution later with sterile water, saline or glucose or an isotonic buffer solution, as appropriate.

Alternatively the formulation may be provided, for example as bags of solution suitable for immediate infusion or vials of liquid ready for injection. Generally ready to use formulations such as these will require storage at less than room temperature and may require protecting from light.

In one embodiment the composition is a vaccine or solution for infusion.

The disclosure also extends to processes of preparing a pharmaceutical composition as defined herein comprising the steps of mixing an antibody or fragment thereof according to the disclosure with a pharmaceutically acceptable excipient.

A suitable dose may be in the range 1ug to 100mg per Kg of patient, such as 1mg to about 10mg per Kg.

In one aspect the disclosure provides an antibody as defined herein or a fragment thereof (or a composition comprising the same) for use as a medicament, for example for the treatment or prophylaxis of a disease caused by VEEV, such as encephalitis.

One aspect provides for use of an antibody or a fragment thereof according to the disclosure (or a composition comprising the same) for the manufacture of a medicament for the treatment or prophylaxis of a disease caused by VEEV, such as encephalitis.

The disclosure also extends to a method of treatment or prophylaxis comprising administering a therapeutically effective amount of an antibody or a fragment thereof, herein described, or a composition comprising the same to a patient such as a mammal, particularly a human in need thereof.

"Treatment" as used herein refers to any therapeutic effect, that is, includes the curing or alleviating of the symptoms of a patient suffering from the disease or infection.

In one aspect of the invention the antibody or fragment thereof is used as a diagnostic, in screening or in activity assays, or in detection of virus in the environment by any assay or transduction system which exploits antibody binding. Alternatively, the antibody may be employed in protein purification.

In one aspect there is provided a kit comprising an antibody or fragment thereof as defined herein.

In the context of this specification "comprising" is to be interpreted as "including".
Aspects of the invention comprising certain elements are also intended to extend to alternative embodiments "consisting" or "consisting essentially" of the relevant elements.

Examples

Materials and Methods

Cells and viruses

The L929 (murine fibroblast), HEK 293 (human kidney) and Vero (simian kidney) cell lines (European Collection of Animal Cell Cultures, U.K.) were propagated by standard methods using the recommended culture media. Stocks of VEEV vaccine strain TC-83 were propagated from a vial of vaccine originally prepared for human use (National Drug Company, Philadelphia, U.S.A.). Strains of VEEV from serogroups IA/B (Trinidad donkey; TrD), IC (P676), ID (3880), IE (Mena II), IF (78V), II (Fe37c), IIIA (BeAn8), IV (Pixuna), V (CaAr508) and VI (AG80) were kindly supplied by Dr. B. Shope (Yale Arbovirus Research Unit, University of Texas, U.S.A.). Virulent virus stocks were prepared and the titre determined as described by Phillpotts (2006). All work with virulent VEEV was carried out under U.K. Advisory Committee on Dangerous Pathogens Level 3 containment. The construction and properties of Replication-defective Adenovirus type 5 containing no VEEV sequence or expressing the VEEV E2 glycoprotein have been described previously (Phillpotts et al., 2005).

Generation of a VEEV-reactive scFv (single chain variable fragment) phage library and conversion of one clone into a monoclonal antibody

Balb/c mice (7-9 weeks old, Charles River, U.K.) were immunised subcutaneously with 10^5 pfu of vaccine strain TC-83. On days 14 and 21, mice were immunised subcutaneously with a mixture of VEEV strains (TrD, P676, 3880, Mena

II, 78V, Fe37c, BeAn8, Pixuna, CaAr508 and AG80; subtypes IA/B, IC, ID, IE, IF, II, IIIA, IV, V and VI respectively), totalling approximately 10^6LD_{50} (approximately 10^6 pfu). Serum samples were taken from the marginal tail vein on day 28 and assayed for an anti-VEEV polyclonal response by ELISA with beta-propiolactone-inactivated TC-83 antigen (Phillpotts et al., 2005). Spleens from five immune mice were removed and processed to extract RNA (TRIzol Reagent, Invitrogen, U.K.) which was then converted to cDNA (SuperScript III Reverse Transcriptase, Invitrogen). Antibody heavy- and light-chain-specific primers were used in a PCR reaction to generate pools of heavy- and light-chain DNA from the cDNA template (Burmester and Plückthun, 2001). Single chain V_L-Linker-V_H constructs (scFv) were then produced using overlap extension PCR with specific single chain primers incorporating a linker region (Burmester and Plückthun, 2001). Purified single chain DNA was digested (Sfi I; New England Biolabs, U.S.A.) and ligated into pAK100 vector (Krebber et al., 1997). Phagemids were electroporated into *E.coli* XL1-Blue (Stratagene, U.S.A.) to produce a library of unique clones. Specificity of the library was increased by two rounds of biopanning (Kontermann, 2001) against beta-propiolactone -inactivated antigen from strain TC-83. Single colonies were isolated from stock obtained from the second round of panning and phage supernatants produced from these clones were assayed by ELISA with beta-propiolactone -inactivated TC-83 antigen and HRP-conjugated mouse anti-phage M13 (Amersham Pharmacia Biotech, U.K.) as the secondary antibody. Absorbance values greater than twice the background level were deemed to be positive. The scFv gene fragments from the positive phagemid clones were amplified using PCR and initially assessed for uniqueness by analysing restriction digest patterns (BstN I; New England Biolabs, U.S.A.). Those clones regarded as unique were analysed by DNA sequencing and compared at the amino acid level for homology. The supernatants from six phagemid clones, containing equivalent bacteriophage titres, were chosen for analysis by ELISA using sucrose density gradient-purified antigen from multiple VEEV strains (TrD, P676, 3880, Mena II, 78V, Fe37c, BeAn8, Pixuna, CaAr508 and AG80) and HRP-conjugated mouse anti-phage M13 as the secondary antibody . The scFv from the clone exhibiting the strongest, most wide-ranging response was converted into a full murine IgG2a kappa antibody (Haptogen, U.K.). This novel

antibody was designated CUF37-2a and a purified stock of the antibody was supplied by Haptogen.

Testing the activity of CUF37-2a in vitro

The ability of CUF37-2a (20ug/ml) to recognise a variety of VEEV strains was tested by ELISA using sucrose density gradient-purified antigen from strains TrD, P676, 3880, Mena II, 78V, Fe37c, BeAn8, Pixuna, CaAr508 and AG80. So that the reactivity could be meaningfully compared, the VEEV antigens used in the ELISA were first examined by SDS-PAGE and scanning densitometry. Each antigen was diluted in coating buffer to contain an equivalent amount of virus glycoprotein. The ability of the antibody to neutralise virus infectivity was also determined. CUF37-2a (25ug) was mixed with VEEV strains TrD, Fe37c or BeAn8 (approximately 100pfu) and incubated at 4°C overnight. Residual infectious virus was estimated by plaque assay in L929 cells.

Assessing the glycoprotein specificity of CUF37-2a

The capacity of CUF37-2a to bind to the VEEV E2 glycoprotein was determined by immunofluorescence staining. HEK 293 cells were infected at a multiplicity of infection (m.o.i.) of 10 with an empty Adenovirus or with an Adenovirus expressing the VEEV E2 glycoprotein. The cells were fixed in acetone after 48h and were incubated with 10ug/ml CUF37-2a followed by a 1/800 dilution of anti-mouse IgG conjugated to FITC (Sigma, U.K.) before being examined under UV illumination.

Determining the in vivo activity of CUF37-2a

The ability of CUF37-2a to protect against a challenge dose of $100LD_{50}$ (approximately 30-50 pfu) VEEV strain TrD (subtype IA/B) was tested. Groups of Balb/c mice (7-9 weeks old, Charles River, U.K.) remained untreated or were injected intraperitoneally with 5, 50 or 100ug of antibody in 50-100ul PBS. The challenge virus was administered subcutaneously 24h later. After challenge, mice were observed twice daily for clinical signs of infection by an independent observer. Humane endpoints were used and these experiments therefore record the occurrence of severe disease rather than mortality. Even though it is rare for animals infected with virulent VEEV and showing signs of severe illness to survive, our use of

humane endpoints should be considered when interpreting any virus dose expressed here as 50% lethal doses (LD_{50}).

Enzyme immunoassay

Mouse sera, harvested by cardiac puncture 14 days after the challenge dose was administered, were assayed for VEEV-specific IgG1 antibodies using sucrose density gradient-purified antigen from strain TrD (Phillpotts, 2006). Immunoglobulin concentrations were estimated by comparison of the absorbance values generated by diluted serum samples (three replicates) with a standard curve prepared from dilutions of mouse IgG1 (Sigma, U.K.).

Titration of virus in the brain

The amount of VEEV strain TrD present within mouse brains was determined by titration on Vero cells. Brains were removed and homogenised in 2ml PBS by passing through a 70um nylon cell strainer (BD Falcon, U.K.). 200ul of the cell suspension were added to each well of the first column of a 96-well plate and the homogenate was then serially diluted (1:10) in cell culture media across the plate. 100ul of the diluted homogenate from each well were then added to the corresponding well of a 96-well plate containing confluent monolayers of Vero cells. The cells were incubated for 72h after which time the monolayers were fixed by the addition of 10% (v/v) formal saline and stained with 0.1% (w/v) crystal violet. The concentration of VEEV, expressed as 50% tissue culture infectious doses ($TCID_{50}$), was calculated by Reed-Muench analysis of virus-positive wells (Butcher and Ulaeto, 2005). The concentration was then converted to pfu by multiplying the $TCID_{50}$ value by 0.69 (Dubois et al., 2004).

Statistical methods

Statistical analysis was performed using the Mantel-Maenszel Logrank test and GraphPad Prism (www.graphpad.com) software.

Results

Generation of a novel VEEV-specific monoclonal antibody

Balb/c mice were initially immunised with VEEV vaccine strain TC-83, which is known to provide solid protection against a large challenge dose of most, if not all, mouse-virulent VEEV strains. Two doses of a mixture of representative viruses from subtypes IA/B, IC, ID, IE, IF, II, IIIA, IV, V and VI were then administered to the immune mice on days 14 and 21. The anti-VEEV immune response was assessed on day 28 (end-point titre greater than 1:500 000) and the spleens removed for extraction of RNA and conversion to cDNA. This was used to create a phage library expressing single chain variable fragments (scFv) which was enriched for antigen-specific scFv by two rounds of panning with antigen from VEEV strain TC-83. Individual phagemid clones were then tested for reactivity to strain TC-83 by ELISA and positive clones were assessed for uniqueness by analysing restriction digest patterns. Eight unique clones were sequenced and compared at the amino acid level for homology. A low level of homology was found between the scFv sequences indicating that the response to VEEV is not oligoclonal. Six of the unique clones were tested by ELISA for reactivity to multiple VEEV strains (Figure 1). Phagemid clone #37 showed the highest level of activity to the widest range of strains and a low reactivity to the negative control antigen. It was therefore chosen for conversion into a murine IgG2a kappa antibody, which was designated CUF37-2a. Murine IgG2a was chosen as the framework as it has equivalent biological and functional activities to human IgG1. The amino acid sequence of the scFv incorporated into CUF37-2a is shown in Figure 2.

Activity of CUF37-2a in ELISA and Neutralisation assays

In order to ensure that the range of VEEV reactivity had been retained during the incorporation of scFv from phagemid clone #37 into CUF37-2a, the antibody was tested in an ELISA using antigens from multiple strains (Figure 3). High levels of reactivity were seen for all strains, with the exception of AG80 (subtype VI) but phagemid clone #37 did not react well with this strain either (Figure 1). However, when the ability of the antibody to neutralise virus infectivity was tested, it was found that CUF37-2a was not able to neutralise virus from subtypes IA/B (strain TrD), II (strain Fe37c) or III (strain BeAn8) (results not shown).

Glycoprotein specificity

VEEV has two major glycoproteins (E1 and E2) that occur on the virus surface as a heterodimer. Antibody reactivity to either protein may be associated with protection against virus challenge. When tested, CUF37-2a reacted with cells expressing the E2 glycoprotein (Figure 4) which indicates that the antibody recognises the viral E2 protein rather than the E1 protein. A recombinant Adenovirus expressing the VEEV E1 glycoprotein alone was not available, so the reactivity of CUF37-2a to the E1 protein could not be tested. The possibility that the antibody recognises a shared epitope cannot be ruled out therefore, although monoclonal antibodies with this property are rare.

Passive protection

Previous work has demonstrated that monoclonal antibodies which possess virus neutralising activity are the most effective at protecting mice from VEEV challenge (Phillpotts, 2006). However, protection *in vivo* is not necessarily associated with the ability of antibodies to neutralise virus (Mathews et al., 1985; Phillpotts, 2006). Functions of the Fc region of the antibody also play a role, principally the capacity to bind to macrophage Fc receptors. It was therefore decided to test the ability of CUF37-2a to protect mice against VEEV strain TrD (subtype IA/B).

In three independent experiments (using the same stock of virus for challenge), the ability of a range of doses of CUF37-2a to protect against VEEV disease was assessed (Figure 5). Untreated mice did not survive the challenge dose and the median time to death was six days. The enhanced survival observed when mice were treated with CUF37-2a was statistically significant compared to untreated mice ($P=0.0043$, $P=0.0001$ and $P<0.0001$ with 5, 50 and 100ug CUF37-2a respectively). The increases in survival rates observed when a larger dose of antibody was administered to mice was not significant ($P=0.1139$), although surviving mice treated with 50 or 100ug CUF37-2a showed no clinical signs of infection whereas all mice treated with 5ug CUF37-2a exhibited some clinical signs. However, the relationship between survival and antibody concentration was significant ($P=0.0371$; Figure 6). From the regression equation, 50% protection was achieved with a dose of 9.15ug CUF37-2a. In short a benefit was observed when the higher dose was given but when statistical analysis was performed this benefit

was not thought to be significant. However, the latter is likely to be due to the fact the sample size on which the statistical analysis was performed was small. It is expected that if a larger population was employed the benefit would be statistically relevant.

The sera of mice that had been treated with 100ug CUF37-2a were tested for VEEV-specific IgG1 by ELISA. The levels of IgG1 were measured in order to distinguish the response induced by the murine immune system and CUF37-2a, which is an IgG2a. All mice generated an immune response to VEEV (mean 232.39ng/ml, 95% confidence interval 106.94ng/ml, n=8). However, it is not known if this response had a role to play in the survival of mice treated with CUF37-2a. The brains of mice that had been treated with 100ug CUF37-2a were also harvested and tested for the presence of virus. No virus was detectable in any of the brains (n=8) whereas brains that were harvested from untreated mice culled 7 days after challenge (n=2) contained 2.967×10^7pfu and 5.368×10^7pfu.

Figure 1

Reactivity of phagemid clones to a wide range of VEEV strains

Supernatants from phagemid clones, containing equivalent bacteriophage titres, were tested by ELISA using antigen prepared from VEEV strains TC-83, TrD, P676, 3880, Mena II, 78V, Fe37c, BeAn8, Pixuna, CaAr508 and AG80 (subtypes IA/B, IA/B, IC, ID, IE, IF, II, IIIA, IV, V and VI respectively). Negative control antigen was prepared from cells that had been mock infected. n=3 for all data points.

Figure 2

Annotated amino acid sequence of scFv CUF37-2a

Nucleotide sequences were edited and translated using Lasergene software (www.dnastar.com). The Pel B leader peptide to direct secretion of the scFv to the periplasm of *E. coli* host cells during heterologous expression is underscored with a dotted line. The cleavage point of this signal peptide is indicated with a block arrow. The presence of a Flag-tag antibody at the N-terminus of the protein is shown with a double underline. The poly-glycine linker joining the V_L and V_H chains of the scFv is underlined with a single solid line. The Framework Regions (FR) and

Complementarity Determining Regions (CDR) within the scFv sequence are indicated with arrows and with shading respectively.

Figure 3

Reactivity of CUF37-2a to multiple VEEV strains

CUF37-2a (20ug/ml) was tested by ELISA using antigen prepared from VEEV strains TC-83, TrD, P676, 3880, Mena II, 78V, Fe37c, BeAn8, Pixuna, CaAr508 and AG80 (subtypes IA/B, IA/B, IC, ID, IE, IF, II, IIIA, IV, V and VI respectively). Negative control antigen was prepared from cells that had been mock infected. n=6 for all data points, 95% confidence intervals are shown.

Figure 4

CUF37-2a reacts with the VEEV E2 glycoprotein

HEK 293 cells were infected (m.o.i. 10) with an empty Adenovirus vector (A) or with an Adenovirus expressing the VEEV E2 glycoprotein (B). 48h later, the cells were fixed and reacted with 10ug/ml CUF37-2a followed by anti-mouse IgG-FITC. A and B show representative fields of view under UV illumination, C and D show the identical brightfield views.

Figure 5

CUF37-2a protects against VEEV disease when administered 24h prior to challenge

In three independent experiments, Balb/c mice (7-8mice/group) remained untreated or were injected with CUF37-2a (5, 50 or 100ug) intraperitoneally. $100LD_{50}$ VEEV strain TrD were administered subcutaneously 24h later. After challenge, mice were observed twice daily for clinical signs of infection and were culled when appropriate using humane endpoints. * P=0.0043, **P=0.0001 and ***P<0.0001, Mantel-Maenszel Logrank test.

Figure 6

Enhanced levels of protection against VEEV by prior treatment with increasing amounts of CUF37-2a

0, 5, 50 or 100ug of CUF37-2a were administered to Balb/c mice by the intraperitoneal route and 24h later the mice were subcutaneously challenged with $100LD_{50}$ VEEV strain TrD. After challenge, mice were observed twice daily for a

period of 14 days and were culled when appropriate using humane endpoints (regression line superimposed, R^2=0.952).

Discussion

This is the first demonstration of a monoclonal antibody, specifically designed to be reactive against multiple VEEV strains, being created using phage display technology and molecular biology techniques. The novel antibody retained the wide ranging activity exhibited by the selected scFv and will prove useful in diagnosis and detection of VEEV. Currently, there are a limited number of monoclonal antibodies available for this type of work (Phillpotts, 2006).

In addition to diagnostic and detection reagents, effective antiviral therapies are also required for VEEV as a vaccine is not currently available. There are no antiviral drugs licensed for the treatment of VEEV infection in humans but monoclonal antibodies are finding increasing application for therapies against other viruses (Marasco and Sui, 2007). Monoclonal antibodies have been shown to be protective in the mouse model of VEEV disease (Hunt et al., 2006; Phillpotts et al., 2002; Phillpotts, 2006) and the purpose of this work was to create a novel antibody able to protect against a wide range of strains. For therapeutic applications, antibodies with virus neutralising activity are most desirable but, unfortunately, CUF37-2a was not able to neutralise the infectivity of multiple subtypes (IA/B, II or III). The antibody was therefore only tested against VEEV strain TrD and it successfully protected mice when administered 24h prior to challenge The protective activity of CUF37-2a may have been due to the ability of the antibody to abort the infection, to prevent spread of virus to the brain or to delay virus replication, giving the host immune response time to respond and control virus infection. It was determined that 50% of mice would be protected from a subcutaneous VEEV challenge when a dose of 9.15ug of CUF37-2a was administered 24h prior to challenge. Previous work (Phillpotts et al., 2002; Phillpotts, 2006) has shown that other VEEV-specific monoclonal antibodies (1A4A-1, 3B2A-9 and 1A3B-7) protect 50% of Balb/c mice against an airborne challenge at doses of 8, 10 and 10ug respectively. Although CUF37-2a was not tested against an airborne challenge, protection induced by this antibody, which was generated using a phage library and molecular incorporation into an IgG2a framework, seems to compare favourably to

protection induced by antibodies generated using classical hybridoma technology (Kohler and Milstein, 1975).

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Claims

1. A functionally active binding fragment of an antibody that binds at least two strains of Venezuelan equine encephalitis virus.

2. An antibody fragment according to claim 1, which is a variable region from a heavy or light chain.

3. An antibody fragment according to claim 1, which is a single chain variable region.

4. An antibody fragment according to claim 1, which is a Fab fragment.

5. An antibody fragment according to claim 1, which is a single chain.

6. An antibody fragment according to any one of claims 1 to 5, which comprises the sequence DYYMN.

7. An antibody fragment according to any one of claims 1 to 6, which comprises the sequence WIGWIDPENGDTEYAPKF.

8. An antibody fragment according to any one of claims 1 to 7, which comprises the sequence EVGRGTSAY.

9. An antibody fragment according to any one of claims 1 to 8, which comprises the sequence KASQDIKSYLS.

10. An antibody fragment according to any one of claims 1 to 9, which comprises the sequence YATTLAD.

11. An antibody fragment according to any one of claims 1 to 10, which comprises the sequence LQHYE.

12. An antibody fragment according to any one of claims 1 to 11 which is recombinant.

13. An antibody comprising a fragment as defined in any one of claims 1 to 12.

14. A pharmaceutical composition comprising a fragment as defined in any one of claims 1 to 12 or an antibody as defined in claim 13, and an excipient.

15. The antibody fragment of any one of claims 1 to 12, antibody of claim 13 or pharmaceutical composition of claim 14, for use as a medicament.

16. The antibody fragment of any one of claims 1 to 12, antibody of claim 13 or pharmaceutical composition of claim 14, for use in treatment of Venezuelan equine encephalitis virus infection and/or prophylaxis against.

17. Use of the antibody of any one of claims 1 to 12, antibody of claim 13 or pharmaceutical composition of claim 14, in the manufacture of a medicament for treating Venezuelan equine encephalitis virus infection and/or prophylaxis against.

18. An assay comprising the antibody fragment of any one of claims 1 to 12, antibody of claim 13 or pharmaceutical composition of claim 14 for detecting the presence of VEEV.

19. A method of treating a patient suspected of having a Venezuelan equine encephalitis infection comprising administering to the patient, the antibody fragment of any one of claims 1 to 12, antibody of claim 13 or pharmaceutical composition of claim 14.

20. A method of protecting a patient from Venezuelan equine encephalitis infection comprising administering to the uninfected patient, the antibody fragment of any one of claims 1 to 12, antibody of claim 13 or pharmaceutical composition of claim 14.

Fig.1.

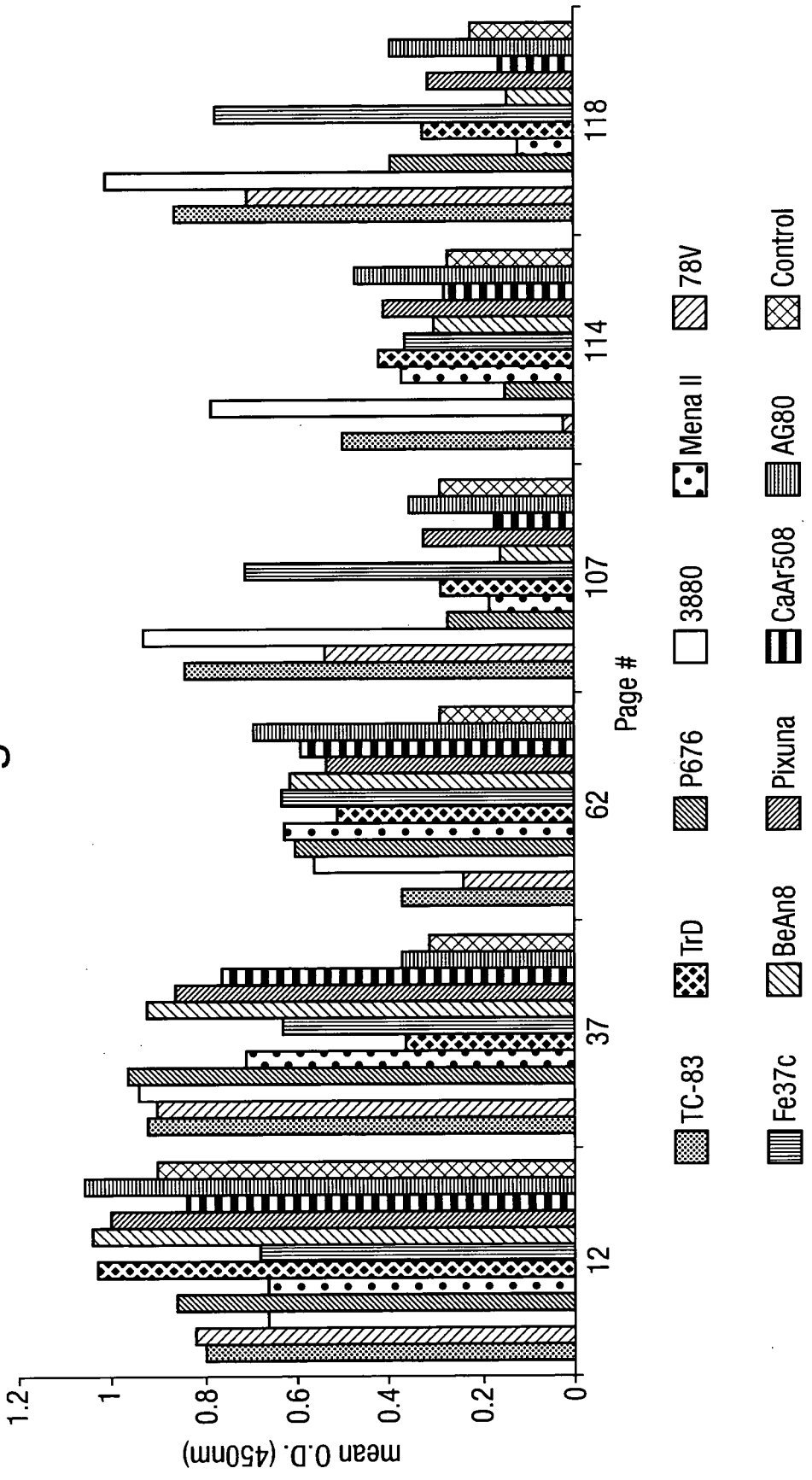
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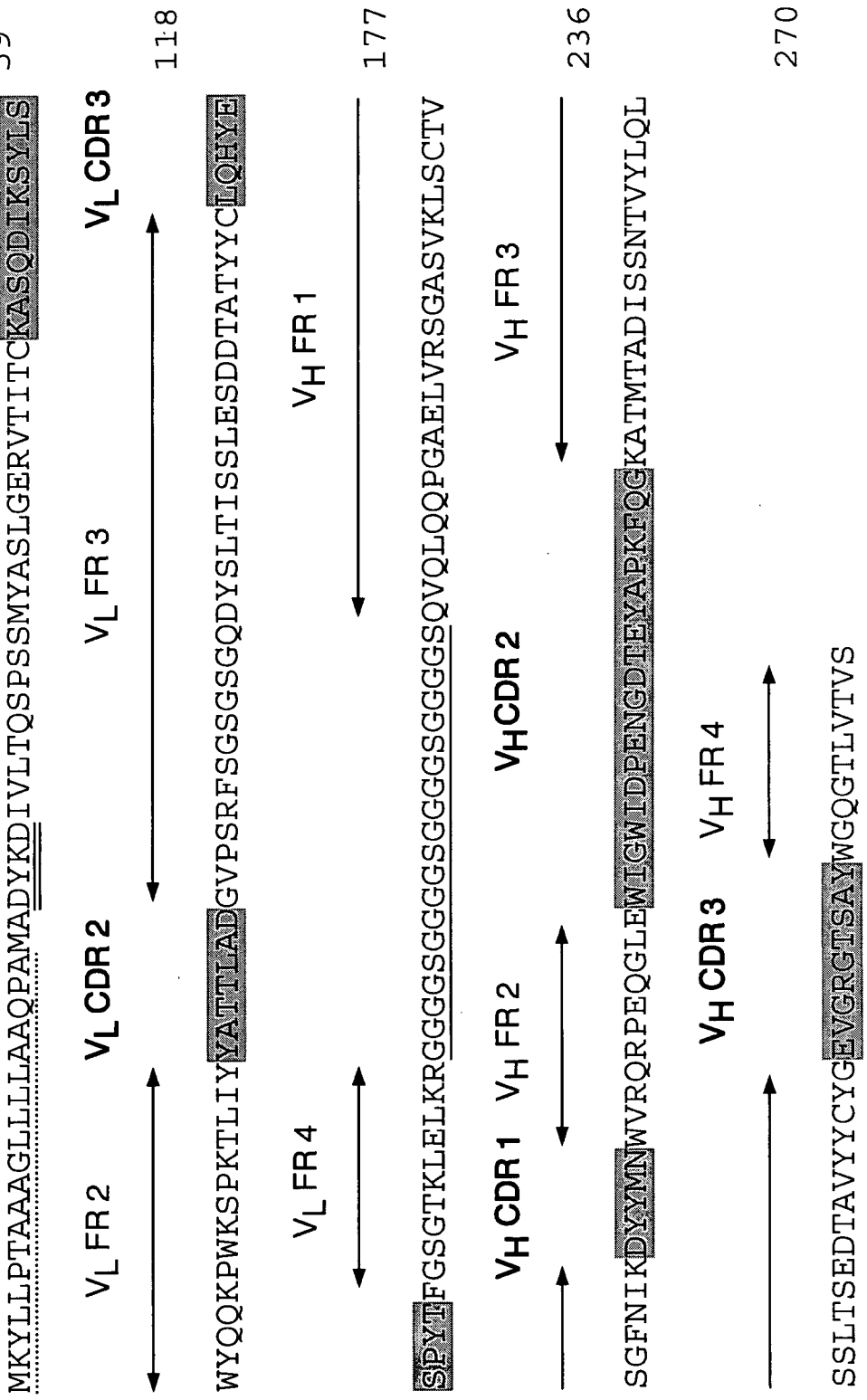
Fig.2.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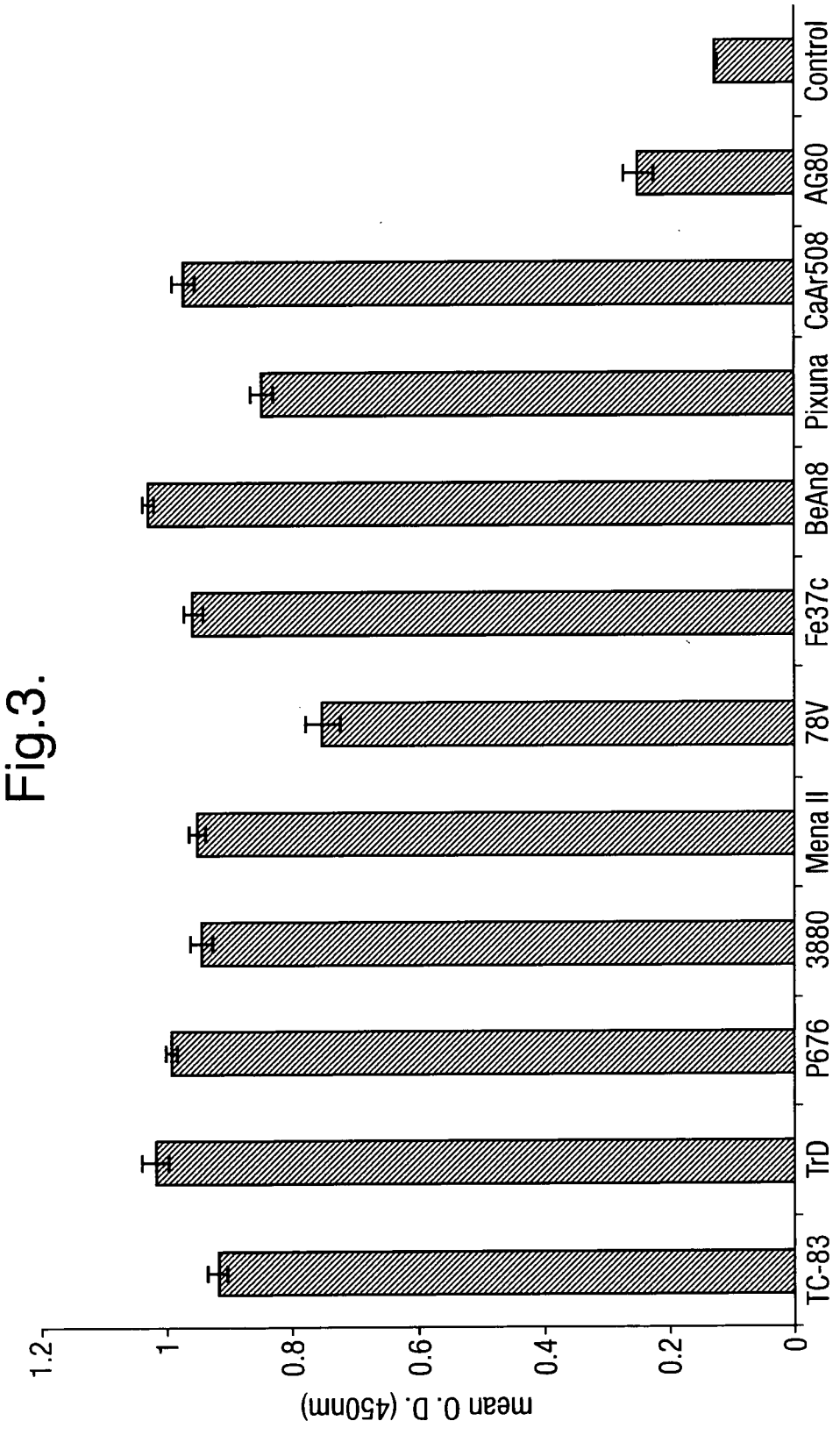
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Fig.4A.

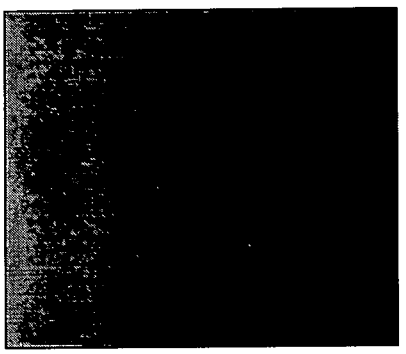
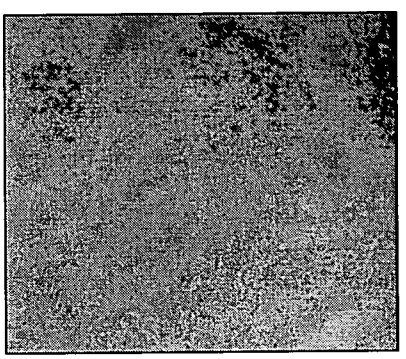


Fig.4B.

Fig.4C.



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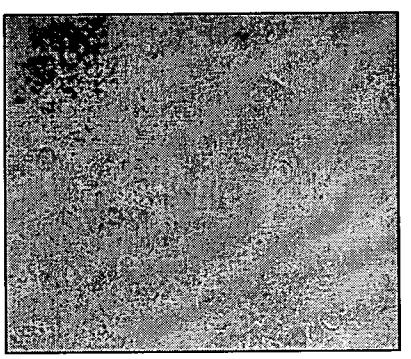

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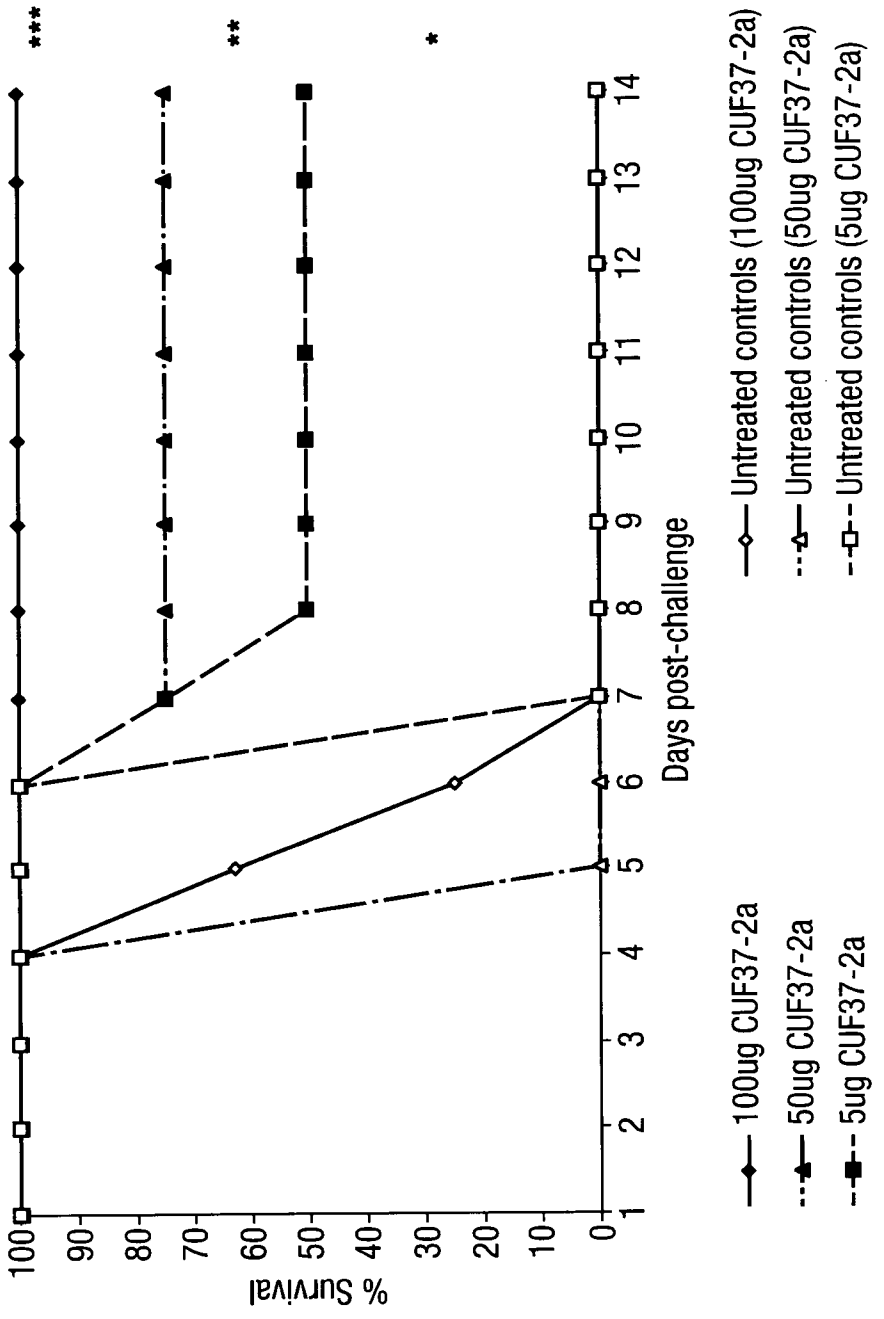
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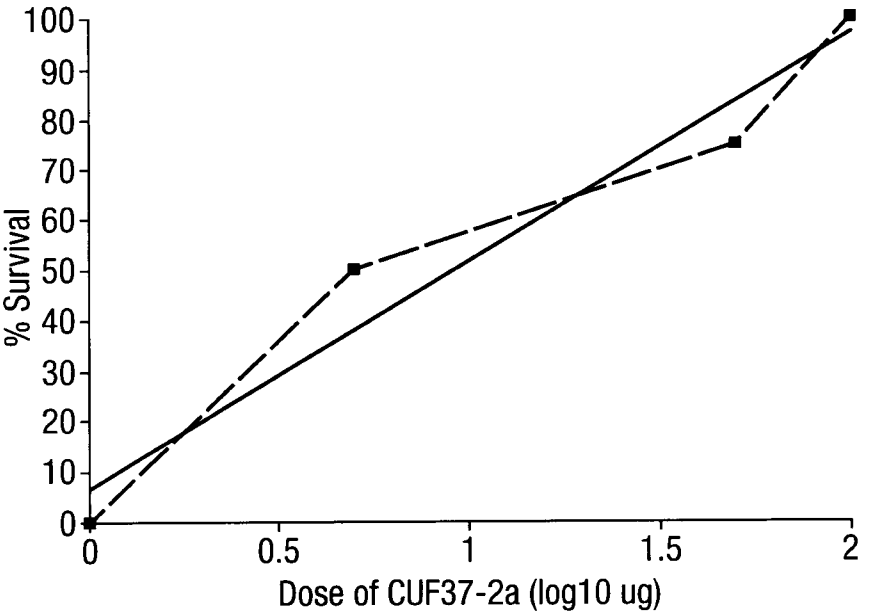
Fig.6.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